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FERMENTATION OF SALTS OF ORGANIC ACIDS AS AN AID TO THE DIFFERENTIATION OF BACTERIAL TYPES

BY

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THE FERMENTATION OF SALTS OF ORGANIC ACIDS AS AN AID TO THE DIFFERENTIATION OF BACTERIAL TYPES.

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(With Plates I and II.)

IN the considerable amount of work already done on the decomposition of the salts of organic acids by bacteria, more attention has been directed to the products of metabolism than to the possibility of differentiating bacterial types by this means.

Koser (1923), in a recent paper on this subject, gives an exhaustive account of the previous work, and shows that no systematic attempt has been made hitherto to apply the results to the differentiation of bacteria.

In the present work it is not proposed to advocate the substitution of organic salts for "sugars" except in those cases in which "sugar reactions" are untrustworthy or fail altogether to differentiate certain serologically well-defined types of bacteria, *e.g.* those of the *Salmonella* group, but we think it reasonable to point out that there are two good reasons for such substitution: (1) the purity of the rarer "sugars" is often doubtful, and (2) the cost of such substances as *inositol* and *trehalose*, a diglucose recommended by Jordan (1923), limits, if it does not actually prohibit their use by investigators. Again, the value of aërogenesis, an important feature of sugar fermentation tests, has been impugned by many workers; Ledingham and Penfold (1915) remark that "many paratyphoid strains give little or no gas in the sugars they normally ferment," and Jordan (1923) states that gas formation in *inositol* media is very variable and therefore an unsafe basis for judgment.

Inositol was employed by Andrewes and Neave (1921) in differentiating the *B. paratyphosus* B and "Mutton" from other types, but they found that one out of twelve "B" types and one out of eight "Mutton" types failed to ferment this substance, and Jordan (1923) also records discrepancies.

It is unnecessary to cite further references here to variations in the fermentation of "sugars" by bacteria, but those interested in this aspect of the question may be referred to the work of Gurney-Dixon (1919) who quotes the important observations of Penfold, Arkwright and many other observers.

Fermentation of organic salts; change in reaction of medium and aërogenesis.

We first investigated the bacterial fermentation of certain organic salts mainly to determine to what extent these reactions could be employed in the differentiation of bacterial types. Salts of the following open-chain acids were used: the basic radicles are shown in brackets after the name and formula of the acid.

(1) *Monocarboxylic acids*

Formic acid H.COOH . (Na, K)
 Acetic acid $\text{CH}_3.\text{COOH}$. (Na, K)
n-Butyric acid $\text{C}_3\text{H}_7.\text{COOH}$. (Na)
n-Valeric acid $\text{C}_4\text{H}_9.\text{COOH}$. (Na)
iso-Valeric acid $\text{C}_4\text{H}_9.\text{COOH}$. (Na)

(2) *Dicarboxylic acids*

(a) Saturated:

Oxalic acid COOH.COOH . (Na_2)
 Malonic acid $\text{COOH.CH}_2.\text{COOH}$. (Na_2)
 Succinic acid $\text{COOH.CH}_2.\text{CH}_2.\text{COOH}$. (Na_2)
 Glutaric acid $\text{COOH}.\text{(CH}_2\text{)}_3.\text{COOH}$. (Na_2)
 Pimelic acid $\text{COOH}.\text{(CH}_2\text{)}_5.\text{COOH}$. (Na_2)

(b) Unsaturated:

Fumaric acid COOH.CH:CH.COOH . (Na_2)
 Maleic acid COOH.CH:CH.COOH . (Na_2)

(3) *Tricarboxylic acid*

Aconitic acid $\text{COOH.CH:C(COOH).CH}_2.\text{COOH}$. (Na_3)

(4) *Hydroxymonocarboxylic acids*

Glycollic acid $\text{CH}_2\text{OH.COOH}$. (Na)
 Lactic acid $\text{CH}_3.\text{CHOH.COOH}$. (Na)

(5) *Hydroxydicarboxylic acids*

Tartronic acid COOH.CHOH.COOH . (Na_2)
 Malic acid $\text{COOH.CH}_2.\text{CHOH.COOH}$. (Na_2)
d-Tartaric acid $\text{COOH.CHOH.CHOH.COOH}$. (Na_2 , Na, K, K_2)
l-Tartaric acid $\text{COOH.CHOH.CHOH.COOH}$. (Na_2)
dl-Tartaric acid (racemic acid) $\text{COOH.CHOH.CHOH.COOH}$. (Na_2 , K_2)
 Mesotartaric acid $\text{COOH.CHOH.CHOH.COOH}$. (Na_2)
 Mucic acid $\text{COOH}.\text{(CHOH)}_4.\text{COOH}$. (Na_2)

(6) *Hydroxytricarboxylic acid*

Citric acid $\text{CH}_2.\text{(COOH).C(OH).(COOH).CH}_2.\text{COOH}$. (Na_3 , K_3)

(7) *Ketomonocarboxylic acid*

Laevulinic acid $\text{CH}_3.\text{CO.CH}_2.\text{CH}_2.\text{COOH}$. (Na)

These salts were used individually in a concentration of 1 per cent. in a basal medium of ordinary nutrient broth having a "reaction" of pH 7.4. Phenol red was employed as an indicator and Durham's tubes to record gas-formation. Sterilisation was accomplished by autoclaving at 115° C. for 20 minutes.

The behaviour of the following organisms on the "salt media" was studied.

4 strains of <i>B. typhosus</i>	1 strain of <i>B. psittacosis</i>
2 „ <i>B. paratyphosus</i> A	2 strains of <i>B. coli communis</i>
3 „ <i>B. paratyphosus</i> B	1 strain of <i>B. coli communior</i>
3 „ <i>B. paratyphosus</i> C	2 strains of <i>B. acidi lactici</i>
3 „ <i>B. enteritidis</i> , Gärtner	1 strain of <i>B. aërogenes</i>
2 „ <i>B. suipestifer</i>	1 „ <i>B. cloacae</i>
2 „ <i>B. aertrycke</i> , Mutton type	1 „ <i>B. pneumoniae</i> , Friedländer
1 strain of <i>B. aertrycke</i> , Newport type	1 „ <i>B. morgan</i> 1
2 strains of <i>B. abortivo equinus</i>	1 „ <i>V. cholerae</i>
1 strain of <i>B. pullorum</i>	1 „ <i>B. gallinarum</i>

The media were sown from fresh well-grown broth cultures, the inoculating dose being a 3.0 mm. loopful of the culture. The tubes were then incubated at 37° C. and daily observations of aërogenesis and changes in reaction were made up to the end of 96 hours.

Of the salts tested very few were found to be readily acted upon by the organisms, and of these few the salts of formic, citric and *d*-tartaric acids were by far the most satisfactory. For the purposes of this paper it will suffice to state briefly the general behaviour of the various organisms on these three salts.

Formates. All the above organisms with the exception of *B. typhosus*, *B. gallinarum*, *V. cholerae* and one strain of *B. abortivo equinus*, formed an abundance of gas and produced a marked change to alkalinity in the medium after 24 hours at 37° C.

Citrates. Trisodium citrate gave very irregular results. A specially prepared chemically pure salt was used; with this *B. paratyphosus* A always showed inhibition of growth and never produced gas or caused any change in reaction. Most of the other organisms, except *B. pullorum*, produced a slight, or rarely a marked, degree of acidity followed after 48 hours by a moderate degree of alkalinity. Apparent gas-formation was unusual and no bacterial strain was found to yield gas constantly; *B. paratyphosus* B, *B. paratyphosus* C, *B. suipestifer*, *B. enteritidis*, Gärtner, *B. aertrycke*, Mutton, *B. aertrycke*, Newport, *B. abortivo equinus*, *B. aërogenes*, *B. cloacae*, *B. pneumoniae* Friedländer, and *B. morgan* I all gave gas on occasions. Gas-formation occurred in 30 per cent. of the tubes inoculated with *B. paratyphosus* C and this represents the most constant result obtained with any organism on this salt.

As will be shown later, apparent gas-formation bears no constant relation to the decomposition of this salt, and changes in reaction during complete decomposition may be very slight.

d-Tartrates. With certain organisms the results obtained with these salts were very constant. *B. typhosus* gave an early and very marked acidity followed after 48 hours by alkalinity. There was no gas-formation. *B. paratyphosus* A gave practically no change in reaction and no gas. *B. paratyphosus* B a slight alkalinity with no preceding acidity and no gas. *B. paratyphosus* C gave a well-marked early acidity changing later to alkalinity and abundant

and constant gas-formation. *B. enteriditis*, Gärtner, Mutton, Newport, *B. suis-pestifer* and most of the other types behaved irregularly.

As a result of more than 4000 observations on reaction and aërogenesis, we found that whereas such changes in reaction as occur are reasonably constant, apparent aërogenesis is an even more variable factor with these salts than with the "sugars." Therefore as the reaction changes alone are insufficient to afford a basis for differentiation we were compelled to abandon the test in this form. Before doing this, however, every effort was made to determine the causes of irregularity in aërogenesis.

The following factors which might possibly influence aërogenesis were investigated:

(a) Method of sterilisation of the medium.

- (1) By autoclaving at 115° C. for 15 minutes.
- (2) By steaming on two successive days for 20 minutes each.
- (3) By filtration through a Doulton germ-proof filter candle.

(b) Reaction of the medium.

(1) The effect of adjusting the medium to various reactions from pH 6.6 to pH 8.0 was investigated.

(2) The medium was used "buffered" and "unbuffered."

(c) The nature of the inoculum.

- (1) Sowing from cultures on solid and in liquid media.
- (2) Sowing from broth cultures of varying age from 3 hours to several weeks.

(3) Variation in the quantity of the inoculating dose.

(d) Variations in the organic salt medium.

- (1) Use of different concentrations of the fermentable salts.
- (2) Employment of a richer basal medium, including the use of different brands of peptone.
- (3) Omission of the indicator.

This formed a very extensive piece of work, which yielded many observations of interest, but, unfortunately, for its main object, the investigation of variability in aërogenesis, it proved fruitless (cf. however p. 8).

In a previous paper on this subject one of the present authors (Brown, 1921) showed that the growth of certain bacteria was inhibited while that of certain others was enhanced by the addition of a soluble citrate to the medium, and that where enhancement of growth occurred, the citrate was decomposed and utilised by the organisms. This fact could be easily demonstrated by the addition of a solution of *lead acetate* to the cultures, when those tubes which showed enhancement of growth yielded a small granular precipitate, while those which showed inhibition yielded a very bulky white precipitate of lead citrate similar to that formed in the control tube.

Koser (1923, 1924), using a large number of strains of the colon-aërogenes

group, has confirmed these observations on the value of inhibition or enhancement of growth in citrated media as a differential test for these organisms. He does not, however, employ the method of precipitation by lead salts.

The question then arises as to whether the precipitation test can yield more consistent results than those obtained by recording acid or alkaline changes and aërogenesis. In this connection a strain of *B. suipestifer*, which gave a feeble fermentation of dulcitol was chosen and plated out. Twelve discrete colonies were picked off into a series of broth tubes, and from these twelve dulcitol tubes were sown. Of the twelve, four showed vigorous fermentation with a copious evolution of gas and the remaining eight were unaffected. These twelve cultures were then tested on different salts of *d*-tartaric acid, but here again gas formation was a variable phenomenon occurring in only seven out of twenty-four tubes, although all showed an acid change followed by alkalinity. When, however, the precipitation test was applied to these twenty-four tubes, decomposition was found to be complete in every case. Examination showed the cultures to be pure, and the agglutination reactions (with *B. paratyphosus* C and *B. glässer* sera) were normal.

Similar observations were made on these and other salts with strains which were notoriously irregular in their reaction changes and aërogenesis, and in all cases the precipitation test was found to yield consistent results.

As further evidence of the superiority of the precipitation method we would cite the fermentation of salts of *l*-tartaric acid. These salts may be completely decomposed by various organisms with no other external indication of the changes taking place than luxuriance of the bacterial growth.

We are satisfied that aërogenesis is not a satisfactory criterion and that determination of decomposition of the salts by the precipitation method is a rational and trustworthy test.

Technique of the Precipitation Test.

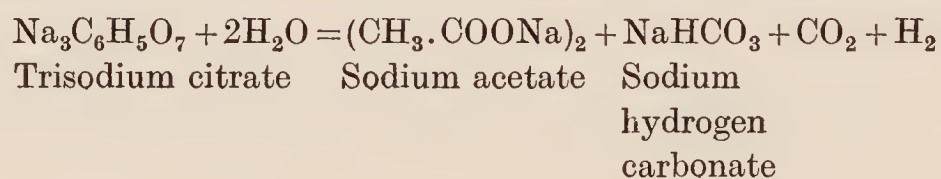
As it was found that commercial meat extracts yield appreciable precipitates with soluble lead salts, it became necessary to abandon nutrient broth as a basal medium, and to employ peptone water. Various commercial peptones were investigated and the most satisfactory results were found to follow the use of "Bactopeptone," which possesses the advantage of being approximately neutral in reaction.

The organic salts are employed in a concentration of 1 per cent. in peptone water (1 per cent. bactopeptone). The reaction of the medium is corrected, if necessary, to *pH* 7.4. The medium is then tubed in 5 c.c. quantities and autoclaved at 115° C. for 20 minutes. Inoculation is made from 24 hours old broth cultures, a 3 mm. platinum loop being employed and the cultures are incubated at 37° C. for 48 hours. For precipitation a saturated solution of lead acetate is employed in the following proportions: 0.4 c.c. is added to 5 c.c. of the citrate medium, and 0.6 c.c. to 5 c.c. of the tartrate or fumarate (p. 12) medium. The tubes are shaken to ensure thorough mixing and are then

replaced in the rack to sediment; they may be examined in a few hours or preferably next day. When decomposition of the salt has not occurred, a voluminous white flocculent precipitate of the lead salt is formed. This precipitate does not settle to the bottom but tends to remain in suspension indefinitely. On the other hand, when decomposition has occurred, a small heavy granular precipitate, made up chiefly of lead carbonate forms, and slowly settles to the bottom of the tube as a small greyish deposit: the colour varying depending on the nature and extent of the impurities (chiefly traces of sulphates and ferric oxide and ferrous sulphide) derived from fermentation of the basal medium.

As the decomposition of citric acid is the most interesting of the series of reactions, a more detailed examination has been made of the products formed from sodium citrate in a typical case, viz. fermentation of sodium citrate by *B. suispestifer*. Apart from traces of the coloured impurities referred to above, only three products were found, viz. acetic acid, carbon dioxide and a trace of succinic acid.

The amount of acetic acid was determined by making 100 c.c. (= an original content of 1 gramme of trisodium citrate) of the reaction liquid, acid, with sulphuric acid and steam-distilling the mixture until 10 c.c. of the distillate did not require more than 0.1 c.c. of *N*/5 barium hydroxide solution for neutralisation, and then titrating the total distillate. The mean of several estimations gave 0.48 gramme of acetic acid per 100 c.c. which corresponds with the formation of two molecules of acetic acid (0.46 gramme per gramme) from one molecule of trisodium citrate, thus



The results, which would be expected to be low, were always somewhat high and this was traced to the action of the organism on the bactopectone solution. A control solution free from citrate, but otherwise prepared like the citrate solution, gave from 100 c.c. an acid distillate corresponding to 0.09 gramme. When the acid distillate from the action of the organism on citrate in bactopectone water is corrected by this amount it will be seen that the average result 0.39 gramme from 100 c.c. is not far from the amount, 0.46 gramme, required by the equation.

The distillate, after neutralisation by baryta, was taken to dryness and the residual salt crystallised in three fractions, which on analysis gave the following results. Barium, per cent. I, 53.88; II, 53.61. The third fraction was converted into the silver salt and this, after recrystallisation from hot water, gave on ignition silver 64.9 per cent. Barium acetate, $\text{Ba}(\text{C}_2\text{H}_3\text{O}_2)_2$, requires Ba 53.81 per cent. and silver acetate, $\text{AgC}_2\text{H}_3\text{O}_2$, requires Ag 64.66. The whole of the salts obtained from the volatile acids were used in this series of determinations so that there is no room for doubt that the sole volatile acid formed is *acetic acid*.

One hundred c.c. of a 1 per cent. solution of trisodium citrate on precipitation by saturated lead acetate yields 1.51 grammes of lead citrate, $\text{Pb}_3(\text{C}_6\text{H}_5\text{O}_7)_2$, dried at 110°C . *in vacuo*. After the action of *B. suipestifer* on a similar solution of sodium citrate in bactopectone water, the lead precipitate from 100 c.c. of solution weighed only 0.6452 gramme, and this contained 7.62 per cent. of carbon dioxide as estimated directly in a Schrotter apparatus, corresponding to 0.049 per cent. of carbon dioxide present in the fermented liquor as sodium bicarbonate. The carbon dioxide in the solution was then estimated independently by precipitation with saturated calcium chloride.

One hundred c.c. of the liquor gave no immediate precipitate with the reagent, but did so on boiling, indicating as was to be expected, that the carbon dioxide was present as sodium bicarbonate. The amount of calcium carbonate formed was 0.1204 gramme.

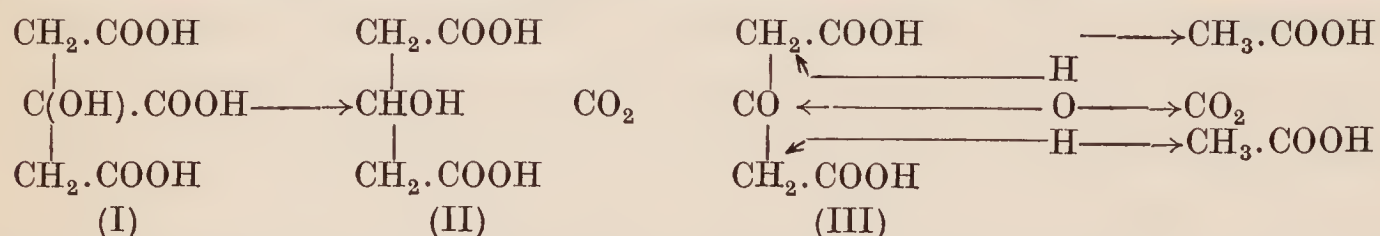
A second 100 c.c. was then made alkaline with ammonia solution and precipitated by calcium chloride solution, yielding 0.2674 gramme of calcium carbonate. These two precipitates were mixed, and carbon dioxide in the mixture estimated: it amounted to 38.7 per cent. Calcium carbonate requires 44.0 per cent. So that the calcium carbonate, like the lead carbonate, contains impurities. This corresponds to 0.103 gramme of carbon dioxide in 100 c.c. of fermented liquor as bicarbonate. The equation given above requires 0.16 gramme. The deficiency is, no doubt, due to the liberation of carbon dioxide from the bicarbonate primarily formed from the citrate by the acids formed by the fermentation of the bactopectone.

A quantity (750 c.c.) of the fermented liquor was distilled to yield 300 c.c. of distillate. The latter was then re-distilled, after making distinctly acid with sulphuric acid, to yield 100 c.c. of distillate. This had specific gravity 0.9993 at $15^\circ/15^\circ$; on re-distillation to yield 50 c.c. the specific gravity fell to 0.996 at $15^\circ/15^\circ$ and the distillate contained traces of insoluble fatty acids. It was then re-distilled, after the addition of potash solution, to yield 30 c.c., which had specific gravity 0.996 at $15^\circ/15^\circ$ and gave a slight iodoform reaction. A blank test with bactopectone water fermented by the same organism, carried out in precisely the same manner yielded a final distillate having specific gravity 0.994 at $15^\circ/15^\circ$, so that whatever the trace of volatile neutral substance produced, causing the fall in specific gravity, may be, it appears to come from the bactopectone and not from the citrate.

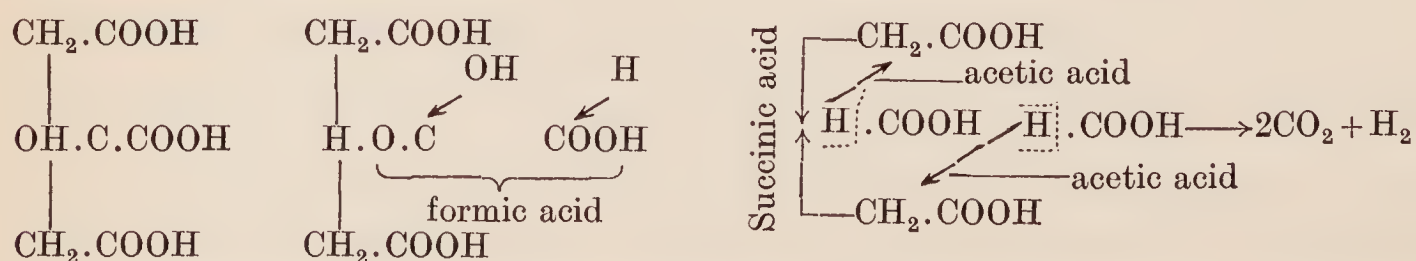
The whole of the mother liquors from the foregoing estimations were kept, de-leaded when necessary, and neutralised. They were then taken to low bulk *in vacuo* until salts, chiefly sodium sulphate from the reagents used, began to crystallise out. The mother liquor from these was made acid with sulphuric acid and thoroughly extracted by agitation repeatedly with ether and acetic ether. The solutions in these solvents were dried over anhydrous sodium sulphate and the solvents distilled off leaving sticky residues, which gradually became crystalline. The residues were mixed, boiled with water till nothing more dissolved, the boiling solution decolourised with charcoal,

filtered and concentrated to low bulk, and left to crystallise. The crystalline acid obtained melted at 180° , had a molecular weight of 120 (if dibasic) as determined by titration with $N/5$ baryta solution, and yielded a beautifully crystalline anhydrous barium salt, which on analysis furnished 92.75 per cent. of barium sulphate. Succinic acid melts at 181° , has a molecular weight of 118, and barium succinate is anhydrous and should yield 92.11 per cent. of barium sulphate. The amount of pure succinic acid obtained from liquors originally containing 10 grammes of trisodium citrate was only 0.0786 gramme, but owing to the greasy and intractable character of the ether and acetic ether residues, it was probably not all recovered.

A control experiment with similar quantities of bactopeptone water fermented by the same organism yielded under like treatment no crystalline acids, though it yielded to ether and acetic ether residues very similar in character to the crude residues obtained from the fermented citrate liquor. It seems clear from these results that sodium citrate (citric acid I) is fermented by *B. suipestifer* in a very simple manner, which might be represented thus:



If this were the case we should have expected to find traces of β -oxyglutaric acid (II) or acetonedicarboxylic acid (III) in the fermentation liquor, but the only acid found apart from the two ultimate decomposition products was succinic acid. In view of this we think it more probable that the group C(OH).COOH in citric acid is attacked at once and as a whole by the organism giving rise by hydrolysis to two molecules of formic acid and momentarily to two $\text{CH}_2\text{.COOH}$ residues. The formic acid is then decomposed by the organism liberating hydrogen and carbon dioxide, the latter partly escaping and partly forming sodium bicarbonate, whilst the hydrogen combines with the CH_2COOH residues to form acetic acid, but the latter reaction is not quite complete, and part of the $\text{CH}_2\text{.COOH}$ residues coalesce to form succinic acid¹, thus:



If this explanation is correct it throws some light on the variability of aërogenesis, since the amount of hydrogen evolved will depend on the amount of

¹ While this paper was being prepared, a paper by Grey (1924) appeared in which a similar explanation is given of the decomposition of citric acid by *B. coli*.

succinic acid formed, and where conditions favour the production of this acid, the amount of hydrogen evolved will be considerable, whereas if the production of acetic acid is favoured the amount of hydrogen liberated will be small.

Salmonella Group.

Owing to the increasing importance of the *Salmonella* group of bacteria, evidenced by the mass of literature, which has accumulated during the past few years, and the present divergence of opinion on the classification of the various members of the group, we considered that it would form suitable material for the trial of this new test. For this purpose we employed the following *Salmonella* strains:

<i>B. paratyphosus</i> A	5 strains	<i>Salmonella</i> , Stanley type	2 strains
<i>B. paratyphosus</i> B	13 „	„ Reading „	1 strain
<i>B. paratyphosus</i> C	11 „	„ Binns „	2 strains
<i>B. suipestifer</i>	12 „	„ “G” „	2 „
<i>B. aertrycke</i> , Mutton type	10 „	<i>B. völdägsen</i>	3 „
„ Newport type	2 „	<i>B. glässer</i>	2 „
<i>B. enteritidis</i> , Gärtner	13 „		

We take this opportunity of expressing our thanks to Dr R. St John-Brooks, Curator of the National Collection of Type Cultures, for his great kindness in supplying us with the majority of these strains, and also to Sir Frederick Andrewes for several strains of *B. suipestifer*.

With the exception of some of the Gärtner strains, all the foregoing *Salmonellas* yielded perfectly constant results when tested on sodium citrate, sodium *d*-tartrate and sodium *l*-tartrate, and it will be shown that these results must prove of considerable help in allocating the various types to their respective positions within the group. The discrepancies shown by the Gärtner strains will be discussed later. The action of each type on these three salts may be summarised as follows:

B. paratyphosus A did not ferment any of the salts mentioned. The cultures therefore after the addition of the lead acetate solution gave a bulky white suspension similar to that in the control tube, indicating absence of decomposition.

B. paratyphosus B. All thirteen strains readily decomposed citrate and *l*-tartrate, but failed to decompose *d*-tartrate. Five strains of this type were grown on *d*-tartrate medium at 37° C. for 28 days, but when tested no evidence of decomposition of the salt could be found.

B. paratyphosus C. The eleven strains of this type differed markedly from *B. paratyphosus* B in their constant failure to ferment *l*-tartrate within 48 hours, while readily fermenting *d*-tartrate. They also fermented citrate.

B. suipestifer. The eleven strains gave reactions similar to those recorded for *B. paratyphosus* C. The same applies to the single strain of type Reading.

Salmonella Type “G.” One strain of this type, and a strain isolated during the course of this work by blood culture from a case clinically resembling enteric fever and identified serologically with Type “G,” were not distinguishable by fermentation of the three salts from *B. paratyphosus* C or *B. suipestifer*.

B. aertrycke, Mutton. The eleven strains constantly decomposed all three salts. A similar result was obtained with the two strains of the Newport type, the two of type Binns, and two of type Stanley.

So far, in dealing with sixty strains of ten *Salmonella* types, we have met with perfectly consistent results, and before passing to the irregular behaviour of the *B. enteritidis*, Gärtner strains, it is convenient to summarise these results.

Organism and number of strains tested	Trisodium citrate	Sodium <i>d</i> -tartrate	Sodium <i>l</i> -tartrate
5 <i>B. paratyphosus</i> A	—	—	—
13 <i>B. paratyphosus</i> B	+	—	+
11 <i>B. paratyphosus</i> C	+	+	—
12 <i>B. suipestifer</i>	+	+	—
10 <i>B. aertrycke</i> , Mutton	+	+	+
2 <i>B. aertrycke</i> , Newport	+	+	+
2 <i>B. aertrycke</i> , Binns	+	+	+
2 Type Stanley	+	+	+
1 Type Reading	+	+	—
2 Type "G"	+	+	—
2 <i>B. glässer</i>	—	—	—
1 <i>B. voldägsen</i> (Damman)	—	—	—
1 <i>B. voldägsen</i> (Wegener)	+	+	+

(+ = decomposition, — = no change)

It has already been stated that these results were obtained after 48 hours' incubation and it may be asked whether any delayed reactions were observed. During the investigation of these sixty strains delayed reactions were observed twice, viz. *B. suipestifer* (Indiana) which failed to decompose citrate in 48 hours but decomposed it in 96 hours, and one strain of *B. aertrycke*, Mutton (Mac-Conkey) which also took 96 hours to decompose citrate.

Plate I shows the results of an actual test with four different *Salmonella* types grown on three of the salt media (trisodium citrate, sodium *l*-tartrate, and sodium *d*-tartrate). It will be seen that those tubes in which decomposition of the salt has occurred show only a small greyish precipitate after addition of lead acetate, while those in which the salt was unaffected show a bulky white suspension.

Irregular behaviour of some Gärtner strains.

We have examined the following thirteen strains: the numbers refer to the catalogue of the National Collection of Type Cultures:

<i>B. enteritidis</i> Gärtner (Bainbridge)	75	<i>B. enteritidis</i> Gärtner (Guinea-pig 158)	252
„ (Limerick)	125	„ (Turner)	305
„ (Newcastle)	126	„ (Rockefeller Inst.)	410
„ (Stokes)	127	„ (Paracoli)	577
„ (McNee)	128	„ (Ratin)	617
„ (Original)	203	„ (Liverpool virus)	618
„ (Danysz)	205		

Of these strains Nos. 127, 203, 252, 305, and 410 decomposed all three salts in 48 hours behaving thus like the *aertrycke* strains. The remaining strains exhibited considerable irregularity in their action on dextro- and laevo-tartrates; sometimes fermenting these salts completely in 48 hours, but more

frequently requiring 96 hours or longer, and occasionally failing altogether. Two strains (126 and 128) rarely attacked these two salts.

When decomposition did not occur, it was noticed that the reaction of the medium became slightly acid and remained so. In complete decomposition an early acid change was always succeeded by a marked alkaline change probably due to the production of sodium bicarbonate from the fermentation of the salt.

Even in simple peptone water these types produce an alkaline reaction in about 48 hours.

Examination showed the cultures of all strains to be pure. Agglutination and absorption tests were normal and specific except with strains 127 and 410, which were "rough." Sugar fermentations were perfectly normal.

The initial reaction of the medium influenced decomposition to some extent. Thus, at an initial reaction of pH 8.0 six of the strains failed to decompose dextro-tartrate in 96 hours, although this reaction is not unfavourable to fermentations by other types; at pH 7.7 three strains failed, and at pH 7.4 only two strains failed. No further adjustment of reaction gave any more favourable result. Attempts were made to enhance the fermenting powers of the "defective" strains by various means, but were not successful. A richer basal medium was employed and various factors, already discussed in connection with irregularity in aërogenesis, which we thought might influence decomposition of tartrates, were examined, but no explanation of the irregular results was forthcoming. One observation is, however, worth recording. The strains which utilised the two salts irregularly or not at all appeared to be of more delicate habit than those which fermented vigorously and constantly: they died out more quickly in culture. When a set of three months old agar cultures of the thirteen strains was examined, it was found that the only ones living were those which constantly fermented *d*- and *l*-tartrates: the irregular fermenters were dead. This inherent defect of the Gärtner strains was not observed in any other type.

In order to extend the range of usefulness of the precipitation test by adding to the number of test salts, all the salts of organic acids previously employed in the tests for aërogenesis and reaction changes were examined to determine which were suitable for the precipitation technique. It was found that the following formed insoluble lead salts similar to those of citric and tartaric acids: malic, malonic, mesotartaric, racemic, pimelic, aconitic, and maleic acids.

Fumaric, succinic, glutaric and glycollic acids give small heavy granular precipitates with soluble lead salts.

Of these salts, malonate, pimelate, succinate, aconitate, glutarate, and glycollate were apparently not readily decomposed by any member of the *Salmonella* group or allied types.

Racemate, which is composed of an equal moiety of dextro-tartrate and of laevo-tartrate, was decomposed to the extent of 50 per cent. by those types which affected either the laevo- or dextro-salt only. It was completely de-

composed by those types which ferment both varieties, and was unaffected by those which ferment neither. Its use was not continued as more information could be obtained by using the dextro- and laevo-salts separately.

Fumarate. The reactions with this salt were of special interest and of some value. Lead fumarate forms a relatively small characteristic precipitate crystallising in slender needles which sinks rapidly to the bottom of the tubes. When, however, a fumarate medium (1 per cent. sodium fumarate in peptone water) was sown with various types of the Salmonella group, it was found on precipitating the cultures after 48 hours' growth that all the tubes showed a voluminous white precipitate, which remained in suspension, occupying more than three-quarters of the liquid column. The appearance of the tubes was therefore the reverse of that seen in the fermentation of the salts hitherto examined; the cultures in which change had occurred giving a bulky white suspension while the control tube showed a small granular precipitate. When the cultures on fumarate were incubated for four days a second phase of fermentation occurred and when precipitation was done at the end of this phase, it was found that the bulky suspension appeared only in the cultures of *B. paratyphosus* A and *B. paratyphosus* C; all the other types giving eventually the slight greyish precipitate consisting chiefly of lead carbonate indicating still further change. The value of this test lies in distinguishing the "C" type from *B. suispestifer*, Type Reading and Type "G."

In view of the interesting character of the action of these bacteria on sodium fumarate, an investigation has been started into the nature of the decomposition products formed. The fermented liquors were examined in the following way, and the results are given in the Table on p. 13.

One hundred c.c. of the fermented liquor were precipitated by excess of saturated lead acetate solution, and the precipitate collected, washed, dried and weighed (line 1). In it the amount of lead (line 2) was determined: it included lead carbonate in all cases. One hundred c.c. of the fermented liquor were made acid with acetic acid, boiled to remove carbon dioxide, neutralised with ammonia and then precipitated with lead acetate and the precipitate collected, washed, dried and weighed (line 3) and the lead in it determined (line 4).

The amount of carbon dioxide present was estimated by adding saturated calcium chloride solution to 100 c.c. of the fermented liquor, previously rendered alkaline with ammonia, and the precipitated calcium carbonate collected, washed, dried and weighed (line 5). As it frequently contained other calcium salts, the carbon dioxide in it was determined (line 6), and from this the percentage of carbon dioxide present in the solution calculated (line 7). These figures are not satisfactory, as the amount of "calcium carbonate" precipitated varied with the time of standing, *e.g.* from 0.2160 gramme in 12 hours to 0.775 gramme in 14 days from *B. aertrycke*, Mutton, 96 hours, liquor. The figures given are for 12 hours. The volatile acids (line 8) were determined by distilling 100 c.c. of the liquor after making acid with sulphuric acid.

Finally, from the mother liquors available from these determinations, the organic acid or acids present (line 9) were isolated by de-leading when necessary, neutralising with soda, concentrating to very low bulk and, after acidifying with sulphuric acid, extracting with ether.

The crude acids so isolated were crystalline, except in the case of *B. paratyphosus* C 90; here they were partly crystalline and only the crystalline portion was used for the examination. These crude acids were neutralised with soda (line 9, I (a)), the solution taken to dryness and boiled with absolute alcohol to obtain a clean sodium salt, which was then converted into the lead salt by precipitation with lead acetate (line 9, I (b)), and from the lead salts the acids were recovered and re-examined (lines 9, II (a), (b), (c)).

	<i>Bacillus aertrycke</i> , Mutton			<i>Bacillus suipestifer</i> Krause 412	<i>Bacillus paratyphosus</i> C 90	<i>Bacillus paratyphosus</i> A
	24 hrs	48 hrs	96 hrs	96 hrs	96 hrs	96 hrs
1. Lead ppt. from 100 c.e. fermented liquor, grammes	1.73	1.37	1.6	0.87	1.6	1.51
2. Lead, % in 1	68.4	68.4	68.1	71.6	66.1	64.6
3. Lead ppt. from 100 c.e. of fermented liquor freed from carbonates, grammes	1.17	0.92	0.32	0.59	0.28	1.15
4. Lead, % in 3	63.4	67.9	68.3	72.3	61.6	71.2
5. Calcium carbonate ppt. from 100 c.e.*	0.084	0.178	0.216	0.301	0.231	0.112
6. Carbon dioxide, % in 5* ...	—	33.0	31.6	43.9	37.0	30.9
7. Carbon dioxide in 100 c.e. of fermented liquor, grammes (Calc. from 6)*... ..	—	0.06	0.07	0.13	0.08	0.03
8. Volatile acids from 100 c.e. expressed as acetic acid, grammes	0.03	0.03	0.03	0.11	0.04	0.04
9. Characters of crude crystalline acids recovered from fermented liquor†						
I. (a) Soda to neutralise, % ...	68.5	60.0	56.3	58.6	64.7	56.8
(b) Lead in lead salt, % ...	63.4	64.6	64.5	64.5	66.4	65.7
<i>Acids as recovered from lead salts</i>						
II. (a) Melting point	291°	Two fractions m.p. 291° and m.p. 285°	181°	181°	181°	285°
(b) Soda to neutralise, %	68.5	66.0	65.7	66.2	—	64.9
(c) Lead in lead salt, % ...	64.8	64.4	—	64.5	65.1	64.3
10. Composition of recovered crystalline acid	fumaric	fumaric	succinic	succinic	succinic	fumaric

* These figures are unsatisfactory as the amount of "calcium carbonate" precipitated varied with the time of standing: the time selected was 12 hours.

† Fumaric acid melts at 291°, requires 68.9 % of soda for neutralisation, and the lead salt contains 64.5 % of lead.

Tartaric acid melts at 170°; racemic acid at 205–6°. Both require 53.3 % of soda for neutralisation, and the lead salts contain 58.3 % of lead.

Succinic acid melts at 181°, requires 67.7 % of soda for neutralisation, and the lead salt contains 64.09 % of lead.

For determining the nature of the acid isolated (line 10 in Table) the quantities of pure acid ultimately obtained were so small that the method of mixed melting points only could be used. It appears from these results that of the four organisms dealt with, all except *B. paratyphosus* A decompose

fumaric acid with the production of carbon dioxide, volatile acids, and succinic acid, but that the decomposition proceeds more slowly than in the case of citric acid. The bacteriological results indicated on the whole that decomposition of fumaric acid probably took place by an initial formation of racemic acid since only those organisms attacked fumaric acid which were capable of decomposing racemic acid partly or wholly, but no evidence of the formation of racemic acid or its components was found. It is not desired to stress this point unduly at this stage since, only small quantities (250 to 500 c.c.) of the fermented liquors have been examined, and it is not easy to isolate tartaric acid from such liquors in presence of fumaric and succinic acids. It may, however, be pointed out that Quastel (1924) has recently investigated the action of *B. pyocyaneus* and *B. fluorescens* on fumaric acid, and shown that no tartaric acid is produced by these organisms. Further, since succinic acid is one of the products of the action of four of the organisms now dealt with on fumaric acid, it seems probable that this is the first product of the action and is formed by direct reduction of fumaric acid thus:



The increase in the bulk of the lead precipitate from the fumarate medium referred to on p. 12 is due to two different causes. When lead fumarate is precipitated in presence of sodium carbonate, the precipitate (mixed lead carbonate and fumarate) no longer shows the character of lead fumarate, but is amorphous and bulky. This occurs with *B. paratyphosus* A. When, in addition, succinic acid has been formed and is present along with fumarate and carbonate, the precipitates are also amorphous and much more bulky than when fumarate or succinate alone is present.

Maleate fermentation has not been completely examined chemically in any case, but the bacteriological observations indicate that it decomposes in a fashion analogous with fumarate. When fermentation does not proceed beyond the stage of conversion of the salt, a bulky white precipitate is formed on the addition of lead acetate, while complete decomposition is indicated by the formation of a small granular precipitate consisting mainly of lead carbonate, and completely soluble with effervescence on the addition of 0.1 c.c. of glacial acetic acid.

Mesotartrate, which yields an abundant precipitate with lead acetate, is readily decomposed by the Mutton, Newport and Binns types and *B. enteritidis*, Gärtner, but not by type Stanley in the first 48 hours. It therefore offers a ready means of differentiating this last type. Two only of the twelve strains of *B. suispestifer* fermented it, but these two strains—Arkansas and Kunzendorf—are unsatisfactory in other respects. All the other types failed to ferment it.

Mucate. This salt forms a very small precipitate^{*} with lead acetate, which is partly soluble in water. On decomposition of sodium mucate by bacteria, sodium bicarbonate is ultimately formed, which, on the addition of lead

acetate yields a small precipitate of lead carbonate. These two precipitates can be easily differentiated by adding a little acetic acid, which dissolves the lead carbonate but not the lead mucate. The technique of the precipitation test must therefore be modified in the case of this salt by the addition of glacial acetic acid to the lead acetate solution: the proportions employed by us are—glacial acetic acid 0.4 c.c. and saturated solution of lead acetate 0.6 c.c. to 5.0 c.c. of the mucate culture. If the mucate is unaltered, a dense turbidity settling down to a small precipitate results, but if decomposition of the salt has occurred, no precipitate follows the addition of the acid lead acetate solution.

The following types constantly decomposed the 1 per cent. sodium mucate medium, *B. paratyphosus* B, Mutton, Newport, Stanley, Binns, Reading and Gärtner. It was not decomposed by *B. paratyphosus* A, *B. paratyphosus* C, *B. suipestifer*, or Type "G." It therefore offers a means of distinguishing the Reading type from *B. suipestifer* and Type "G." We observed no irregularities in the fermentation of this salt by the various types.

It will be seen from the foregoing that with the use of six salts: citrate, *d*-tartrate, *l*-tartrate, *m*-tartrate, fumarate, and mucate, we have succeeded in obtaining seven different cultural groupings from the eleven different *Salmonella* types. The types we have still failed to differentiate are Mutton, Newport and Binns from one another, and *suipestifer* from Type "G."

The following table gives a summary of the reactions with these six salts, and it is convenient at this stage to compare these results with those hitherto obtained from "sugar" fermentations.

	Citrate	<i>d</i> -tartrate	<i>l</i> -tartrate	<i>m</i> -tartrate	fumarate	mucate
<i>B. paratyphosus</i> A	—	—	—	.	.	—
<i>B. paratyphosus</i> B	+	—	+	.	.	+
<i>B. paratyphosus</i> C	+	+	—	.	—	—
<i>B. suipestifer</i>	+	+	—	.	+	—
<i>Salmonella</i> type "G"	+	+	—	.	+	—
Type Reading	+	+	—	.	+	+
Type Mutton	+	+	+	+	.	+
Type Newport	+	+	+	+	.	+
Type Binns	+	+	+	+	.	+
Type Stanley	+	+	+	—	.	+
<i>B. enteritidis</i> , Gärtner	+	±	±	±	+	+

Results were taken at the end of 48 hours' incubation at 37° C. except in the case of fumarate, in which the incubation was prolonged to 96 hours to allow for its slower decomposition. A + sign indicates complete decomposition of the salt as evidenced by the formation of only a small precipitate consisting chiefly of lead carbonate on the addition of the lead acetate solution. A — sign indicates failure to decompose as evidenced by a bulky precipitate of the lead salt of the undecomposed acid. In the case of fumarate a — sign indicates change of the salt as indicated by a bulky precipitate on addition of lead acetate (cf. p. 12) while a + sign expresses further change as shown by a small precipitate of lead carbonate. For the modified precipitation technique applied in the case of mucate, see above.

The grouping of the types from the above table is as follows: (1) *B. paratyphosus* A, (2) *B. paratyphosus* B, (3) *B. paratyphosus* C, (4) *B. suispestifer* and Type "G," (5) Reading, (6) Mutton, Newport, Binns and Gärtner?, (7) Stanley.

Differential "Sugar" Fermentations of the Salmonellas.

	Xylose	Arabinose	Dulcite	Inosite
<i>B. paratyphosus</i> A	—	+	+	—
<i>B. paratyphosus</i> B	+	+	+	+
<i>B. paratyphosus</i> C	+	+	+	—
<i>B. aertrycke</i> , Newport	+	+	+	—
<i>B. aertrycke</i> , Mutton	+	+	+	+
<i>B. enteritidis</i> , Gärtner	+	+	+	—
<i>B. columbensis</i>	+	+	+	—
<i>B. suispestifer</i>	+	—	—	—

(Andrewes and Neave, *B.J.E.P.*, 1921.)

These "sugar" reactions give us four groups: (1) *B. paratyphosus* A, (2) *B. paratyphosus* B and Mutton, (3) *B. paratyphosus* C, Newport, Gärtner, and *B. columbensis*, (4) *B. suispestifer*.

It possesses an advantage over the organic salts table in differentiating Mutton from Newport, and if the two tables are combined we are presented with eight groups.

We are not aware of the reactions of the newer types Stanley, Binns, Reading, and "G" with the rarer "sugars" nor are we in a position to determine these, but we have no information that they would be helpful in differentiating these types from one another or from other types.

The optimum time limit of incubation.

Although we have shown that six strains of *B. paratyphosus* B taken at random failed to decompose *d*-tartrate after 28 days' incubation, this does not hold good in the case of the laevo-salt when acted upon by *B. paratyphosus* C, for, although no apparent decomposition occurs within the first 48 hours, several strains of this organism were found capable of fermenting this salt when incubation was prolonged. This is directly supported by the observations of Pasteur, who showed that when *Penicillium glaucum* was grown in solution of ammonium *dl*-tartrate the salt of the dextro-acid was destroyed and of the laevo-acid remained; if, however, the decomposition was allowed to proceed further, the laevo-salt was also destroyed. It is obvious, therefore, that when using laevo-tartrate a definite time limit must be adhered to, and we have found 48 hours the most suitable.

Although prolonged incubation does not affect the value of the test in the cases of citrate and *d*-tartrate, for the sake of uniformity we advise a period of 48 hours, which, in practically all cases, is sufficient; this also applies in the cases of mesotartrate and mucate, but with fumarate, in which the action occurs more slowly and may take place in one or more stages (see p. 14), it is necessary to prolong the incubation to 96 hours.

Combinations of fermentable substances.

Various mixtures of salts or of salts and sugars were examined. One of the most useful of these was a mixture of sodium citrate 1 per cent. and glucose 1 per cent., which was fermented by all of the group except *B. paratyphosus* A and C, *B. suipestifer* and Type "G," giving results similar to those obtained with sodium mucate, for which salt it might be substituted in an emergency.

The action of two organisms, viz. *B. paratyphosus* C 90 and *B. aërogenes*, on this mixture was investigated and compared with their action on the two components, sodium citrate and glucose separately. For this purpose the volatile acid (acetic acid) and the reducing power (glucose) were determined in the fermentation liquors; the results are given in the following table. The figures are for 100 c.c. of the fermented liquor in each case.

Organism	Medium	Glucose % (from reducing power)	Volatile acid %, distillation and titration	Glucose % decomposed
<i>B. aërogenes</i> 24 hours	1 % glucose,	0.32	0.15	0.68
	1 % bactopectone			
	1 % sodium citrate,	0.15	0.62	0.85
	1 % glucose,			
	1 % bactopectone			
<i>B. paratyphosus</i> C. 90 24 hours	1 % sodium citrate,	Nil	0.76	—
	1 % bactopectone			
	1 % glucose,	0.70	0.07	0.30
	1 % bactopectone			
	1 % sodium citrate,	0.31	0.13	0.69
	1 % glucose,			
	1 % bactopectone			
	1 % sodium citrate,	Nil	0.26	—
	1 % bactopectone			

It is clear that with both these organisms more glucose is decomposed in presence of sodium citrate, whilst the decomposition of the citrate (as measured by the volatile acid produced) is retarded in presence of glucose. For a full explanation of these differences it will probably be necessary to determine the nature and amounts of the products formed by the action of the two organisms on glucose and citrate separately, but it seems possible that the differences are due to (1) increase in the nutritive material available leading to enhanced growth of the organisms, (2) preference of the organisms for glucose as a nutritive material, (3) alteration in the pH of the medium due to the fermentation products of glucose, which would tend to diminish the formation of acid products, *e.g.* the acetic acid produced by the decomposition of citric acid.

The addition of glucose to the dextro-tartrate medium appeared to prevent the decomposition of this salt by any member of the Salmonella group. The reasons suggested for the retarded decomposition of citrate by *B. aërogenes* and *B. paratyphosus* C 90 in presence of glucose probably also apply in this case, the similarity in decomposition products possibly having special importance in this instance.

Various mixtures of two or of three salts, giving a total concentration of 1 per cent. were tried, and in some cases yielded interesting results which,

however, need not be detailed here, as they do not bear on the main question of differentiation.

In many instances combinations of two salts which individually were readily fermented by an organism gave a mixture which resisted decomposition by this organism. In other cases a salt not normally fermented by a certain strain was rendered susceptible to decomposition by the addition of a readily fermented salt.

The results obtained were so varied that at one time it was hoped by the use of such mixtures distinctive tests could be found for every type of the *Salmonella* group, and other groups, but although it was found that the reactions with individual strains of a type were remarkably constant, when several strains of a type were tried divergent results were obtained, and this form of test had to be abandoned. As an example of the use of "mixtures." Sodium formate and sodium citrate are both readily decomposed by *B. paratyphosus* C. If, however, these salts be combined in the proportion of 0.5 per cent. each, a strain of *B. paratyphosus* C will apparently still decompose the formate fraction but not the citrate. If, however, 0.5 per cent. sodium racemate be added to the formate-citrate mixture, complete decomposition of all three salts by this strain of *B. paratyphosus* C occurs.

We think these apparent anomalies are also explicable on the lines suggested above, but until we have examined the fermentation products in each case we prefer not to attempt a detailed explanation.

*The correlation of luxuriance of growth of the organism
with decomposition of the salt.*

In the case of citrate, it has been shown (Brown, 1921) that decomposition and utilisation of the salt are associated with enhancement of growth of the organism, and that failure to utilise the salt is associated with a definite inhibition of growth. This enhancement or inhibition of the growth of different types by citrate has also been employed by Koser (1923, 1924) in his tests for the identification of the bacterial types found in domestic water supplies. The test, although not so distinctive as lead precipitation, is trustworthy enough when restricted to citrate, *d*-tartrate and one or two other salts which undoubtedly inhibit the growth of types incapable of decomposing them, but it cannot be depended upon with all salts as in many cases failure to decompose the salt is not associated with any apparent inhibition, and the utilisation of the salt does not in all cases lead to obvious enhancement of growth.

Practical application of the precipitation method.

In order to test the reliability of this means of differentiating the various members of the *Salmonella* group, Dr Schütze very kindly gave us eleven cultures which had presented certain difficulties as regards their serological classification, and the results which we obtained after 96 hours' incubation are shown in the following table.

Organism	Trisodium citrate	Sodium <i>dextro</i> -tartrate	Sodium <i>laevo</i> -tartrate
Kruse	+	+	+
Shanks	+	—	+
Lister	+	+	+
Mudd	+	+	—
Leeds	+	+	—
Edinburgh	+	+	+
<i>Aertrycke</i> Bainbridge	+	+	+
<i>Aertrycke</i> Kral	+	+	+
<i>Aertrycke</i> Lab.	+	+	+
Piper I	+	—	+
Piper II	+	—	+

A 96 hours' incubation was used in this case, as it was found that two cultures, viz. Kruse and Edinburgh, failed to decompose citrate in 48 hours. From these results it will be seen that Shanks, Piper I and Piper II gave the reactions of *B. paratyphosus* B; Mudd and Leeds behave similarly to *B. paratyphosus* C, *B. suipestifer* or Type "G," and that the remainder correspond to *B. aertrycke*. These results entirely agree with Dr Schütze's serological diagnosis as far as it was able to be established.

Two of the above strains are of special interest, viz.: (1) The organism Kruse was found by Dr Schütze (1920) to belong, by virtue of its agglutinogenic and absorptive properties, to a very definite *aertrycke* group, but differed from all its fellow members in not reducing fuchsin and not producing sulphuretted hydrogen in lead acetate medium; also it did not produce gas in dulcitate, however; on the organic salts it behaved as an *aertrycke*. (2) Piper I. When studying this organism, Dr Schütze (1920) found that it was only after preparing a Piper I serum and carrying out an inverse or mirror absorption test that the fact was revealed that it was a true *B. paratyphosus* B, and as we have seen with the organic salts, it behaves as such. So far we have confined our attention to the results obtained with the Salmonella group and we will now briefly discuss the value of this test in differentiating other bacterial groups.

Vibrio cholerae and allied vibrios.

Brown (1921) showed that the character of the growth in citrated broth was an easy way of distinguishing the vibrios of Metchnikoff and Finkler-Prior from the cholera vibrios pathogenic to man.

By applying the precipitation test we have found that the following vibrios readily decompose trisodium citrate, viz. *V. cholerae*, *V. El tor*, *Paracholera* A and B (Mackie and Storer), *Vibrio K.* and *Vibrio Forrest*, whereas the vibrios of Metchnikoff and Finkler-Prior have apparently no action on this salt.

The colon-aërogenes group.

Reference has already been made to the extensive work of Koser (1923-24) on the action of a very large number of strains of this group on citrated media.

We have found that with the strains which we have used both *B. lactis aërogenes* and *B. cloacae* are capable of readily decomposing citrate, thus

differing from *B. coli communis*, *B. coli communior* and *B. acidi lactici*, which fail to attack this salt.

The only other organic salt which we have found useful with this group is sodium mucate, which is apparently not decomposed by *B. cloacae*, but is by all the other members of this group.

The diphtheria group.

The only salt which enabled us to distinguish Hofmann's bacillus from the Klebs Loeffler's bacillus was sodium fumarate; with this salt the former organism, on the addition of lead acetate, yielded a precipitate similar to that in the control tube, whereas in the case of both virulent and avirulent diphtheria cultures a considerably larger precipitate was produced.

B. mallei and B. whitmori.

It was more especially when using sodium fumarate that differences were observed between the growth of these two organisms; two strains of the former and four of the latter were used.

B. mallei on this medium showed a very feeble growth, but in the case of *B. whitmori* a very turbid growth appeared in 24 hours and pellicle formation was marked in 48 hours. It should be mentioned that the four strains of *B. whitmori* had become rugose in character.

The precipitation test demonstrated a certain amount of decomposition of this salt by *B. whitmori*, but no such change took place with *B. mallei*.

Plate II shows the difference in growth of these two organisms on this medium.

The action of B. typhosus on organic salts.

Somewhat conflicting results have been obtained when studying the behaviour of eight strains of *B. typhosus* on these organic salt media.

Three of the eight strains failed to decompose either citrate or dextro-tartrate, two of them fermented both these salts, and two failed to decompose citrate but decomposed dextro-tartrate.

It is interesting to note in this connection the observations of Mandelbaum (1912), who obtained a bacillus from the blood or faeces of more than fifty patients with clinical typhoid fever in Munich, which he named *B. metatyphi*. This bacillus resembled *B. typhosus* in all respects except that it produced alkali instead of acid in media containing glycerol.

With the strains of *B. typhosus*, which we investigated, the three which failed to ferment either citrate or dextro-tartrate produced only a trace of acidity in glycerol after 96 hours' incubation, whereas those which fermented both these salts rendered the glycerol medium distinctly acid in 24 hours. Further it is interesting to note that the three strains which failed to decompose these salts and had a very sluggish action on media containing glycerol were all obtained from the same epidemic and were all mild cases of enteric fever. Each of the eight strains was plated out and twelve colonies picked off into

broth. The resulting cultures behaved on the organic salt media in every respect as the parent strains. Agglutination and reciprocal absorption tests showed no differences amongst any of the strains except in intrinsic agglutinability.

Examination of other allied types.

We have failed to distinguish between *B. melitensis* and *B. abortus* (Bang.) and between *B. pestis* and *B. pseudotuberculosis rodentium*.

SUMMARY AND CONCLUSIONS.

(1) Reaction changes and production of gas in organic salt media are not sufficiently constant to form a reliable diagnostic criterion for the differentiation of bacterial types.

(2) The enhancement or inhibition of bacterial growth in such media in the majority of cases bears a direct relationship to the utilisation of the salt by the organism. This furnishes a useful differential test for certain organisms when citrates are used, but cannot be applied in the cases of all salts.

(3) The bacterial decomposition of the salts of those organic acids which form insoluble lead salts can be clearly demonstrated by the addition of suitable quantities of a solution of lead acetate to the culture.

(4) By the use of six organic salts, seven different groupings of the common Salmonella types can be obtained, whereas the sugar reactions have, up to the present, yielded only four different groupings.

(5) Regarding other groups of bacteria, the organic salts form an easy means of distinguishing between pathogenic and certain non-pathogenic vibrios, and between certain of the members of the *coli-aërogenes* group, and also between *B. diphtheriae* and Hofmann's bacillus, as well as between *B. mallei* and *B. whitmori*.

(6) The six organic salts employed in this test are relatively inexpensive, will stand sterilising by autoclave, and can be obtained with certainty in a state of purity much more readily than the rarer "sugars."

(7) The nature of the decomposition products of citric acid has been fully examined in the case of *Bacillus suispestifer*; it has been shown that the products are acetic acid, carbon dioxide and succinic acid, and a simple explanation of the mechanism of this reaction is put forward. In the case of fumaric acid a preliminary examination shows that the acid is converted into succinic acid probably by direct reduction. Maleic acid appears to behave in an analogous manner to fumaric acid. Further work on these acids is in progress.

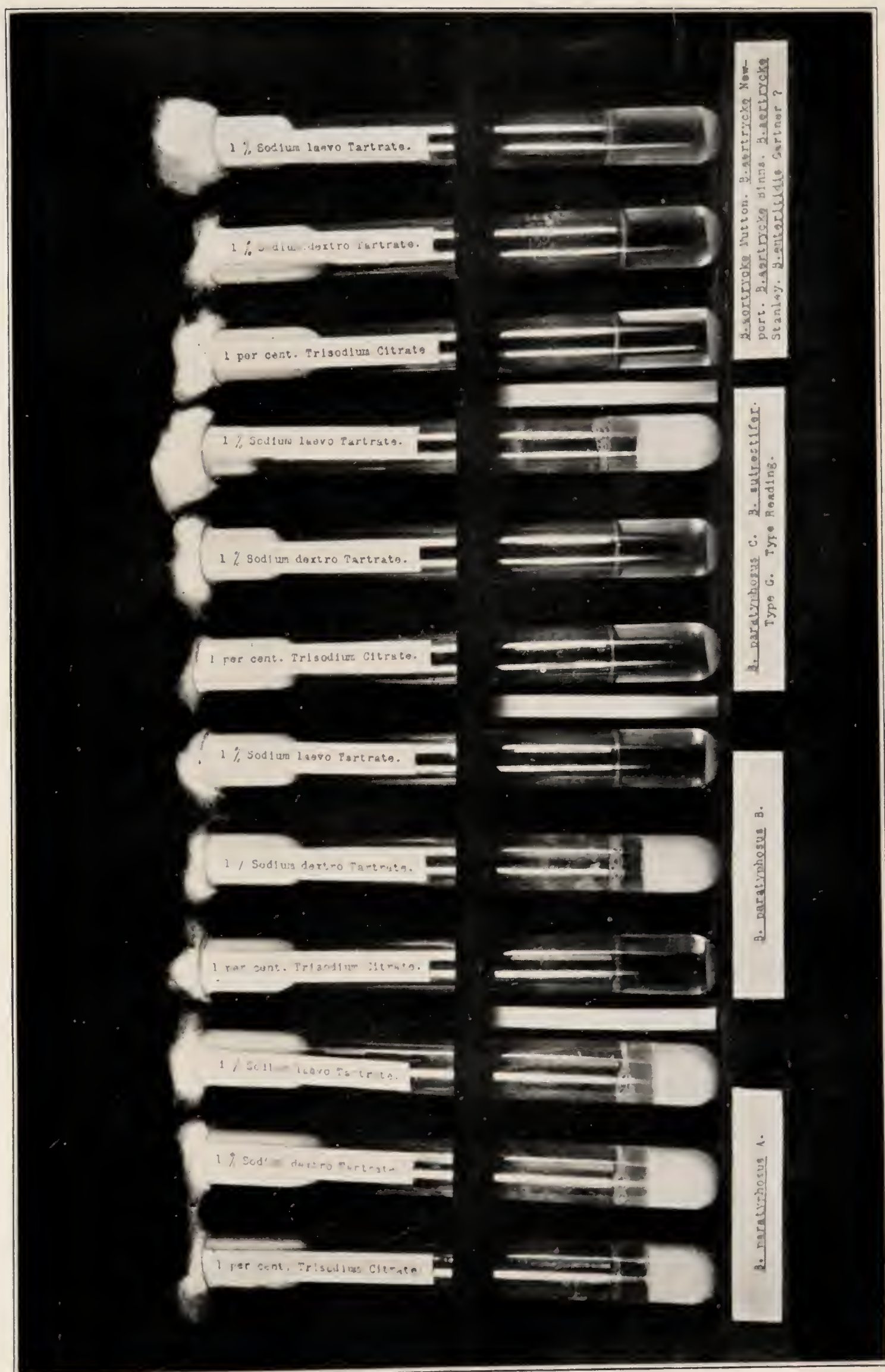
(8) A large number of organic acid salts have been tried, but only the six suggested have given useful results. It appears that simple aliphatic monobasic and dibasic acids, with the exception of formic acid, are not decomposed readily by the bacteria investigated, and this is also true of monohydroxycarboxylic acids. Readiness of decomposition is first shown by the dihydroxydicarboxylic acids (tartaric acids), and appears to be at its best in the hydroxytricarboxylic acid (citric acid).

(9) While organic salt fermentation tests have been found particularly useful in the cases of the bacterial groups dealt with in this paper, they cannot be substituted for the "sugar reactions" in general use.

REFERENCES.

- ANDREWES, F. W. and NEAVE, S. (1921). *British Journal of Experimental Pathology*, II. 157.
BROWN, H. C. (1921). *Lancet*, I. 23.
DIXON, G. (1919). *The Transmutation of Bacteria*, p. 50. Cambridge University Press.
GREY, E. C. (1924). *Proceedings of the Royal Society*, XCVI. 156.
JORDAN, E. O. (1923). *Journal of Infectious Diseases*, XXXIII. 567.
KOSER, S. A. (1923). *Journal of Bacteriology*, VIII. 493.
—— (1924). *Ibid.* IX. 59.
LEDINGHAM, J. C. G. and PENFOLD, W. J. (1915). *British Medical Journal*, II. 704.
MANDELBAUM, M. (1912). *Centralbl. f. Bakteriol.* 1 Abt. Orig. LXIII. 46.
QUASTEL, J. H. (1924). *Biochemical Journal*, XVIII. 365.
SCHÜTZE, H. (1920). *Lancet*, I. 93.
TOPLEY, W. C. C., WEIR, H. B. and WILSON, G. S. (1921). *Journal of Hygiene*, XX. 227.









1 % Sodium Fumarate.
1 % Bactopeptone. 48 hours culture.



